

**Activity of Mammalian Cytochrome P450 Reductase from its
Isolated Flavin Domains**

A Senior Honors Thesis

**Presented in Partial Fulfillment of the Requirements for
Graduation with Distinction in Biochemistry in the
Undergraduate Colleges of the Ohio State University**

By

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Dedicated to my family.

Acknowledgment

The completion of this project would not have been possible without the help of many people. First, I would like to thank my advisor Dr. Richard P. Swenson for his guidance and excellent design of this good project. I also would like to thank the Department of Biochemistry for giving me the opportunity to do research through the Summer Undergraduate Research Program. Without this experience, I would not have been off to a good start for this project nor would I have gained a lot of experiences. Lastly, I would like to thank all the past and present lab mates for their guidance, and also their support and dedication that made my stay in the lab a great experience.

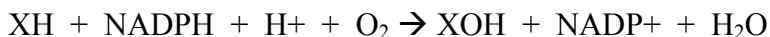
Abstract:

The goal of this project was to purify the isolated FAD domain from rat cytochrome P450 reductase and to study its electron transferase activities, either alone or in combination with added isolated FMN domains from various sources. The rat FAD domain was purified by affinity chromatography on immobilized 2',5'-adenosine diphosphate. The purified FAD domain was observed to retain the NADPH-dependent ferricyanide reductase activity exhibited by the intact reductase indicating that its electron transferase activity remains intact. Cytochrome *c* (an analog for cytochrome P450) reductase activity similar to the intact enzyme could be reconstituted by mixing the isolated rat FAD and FMN domains *in vitro*; however, evidence for a stable 1:1 complex was not obtained. Instead, the activity was linearly dependent on the ratio of the FMN:FAD domains suggesting an alternate electron transfer mechanism. The rat FAD domain was mixed with homologous FMN-binding domains/proteins from other sources to study the flavin domain specificity. Cytochrome *c* reductase activity can not be reconstituted using the structurally homologous flavodoxin; however, somewhat surprisingly, higher levels of activity were obtained using the FMN domain from a related bacterial cytochrome P450 system which displays different redox properties. Cytochrome *c* reductase activity was inhibited at high concentrations of the cytochrome in both the bacterial and rat cytochrome P450 reductase. The mechanism of inhibition is not fully understood but appears to be the result of the formation of a relatively stable complex with the FMN-binding domain. These data support a mechanism for cytochrome reduction involving a requisite movement of the flavin domains in the intact cytochrome P450 reductase enzyme and the formation of a complex with the cytochrome.

Introduction

Cytochrome P450 Reductase: Cytochrome P450 reductase is a member of a superfamily of monooxygenases. It is a crucial enzyme system involved in detoxification in mammals. It participates in the metabolism of compounds such as steroids, fatty acids, prostaglandins, therapeutic drugs, carcinogens, plant natural products, and a variety of toxic compounds. Cytochrome P450 reductase cleaves molecular oxygen, producing an oxygenated product and one equivalent of water. It metabolizes these substrates by hydroxylation. These are the various types of oxygenation reactions that are catalyzed by this system.

Monooxygenase Activity (major):



Oxidase Activity (minor):



Reductase Activity (minor):



There is also an interest to the pharmaceutical industry because it is one of the major enzyme systems used in xenobiotic transformation, that is, in the activation and/or detoxification of drugs. For example, cytochrome P450s are being targeted for drug development for skin diseases. Cytochrome P450s participate in the metabolism of therapeutic drugs, fatty acids, and other compounds. In one skin disease such as psoriasis, the cytochrome P450s are elevated. Some studies show that vitamin D is a natural product in the skin and its biological function is dependent on cytochrome P450 enzymes. 1,25-Dihydroxyvitamin D helps direct a series of vitamin

D-dependent actions with calcium homeostasis, growth, and the immune response. The degradation of vitamin D is the regulatory multi-catalytic CYP24 enzyme that helps introduce C-24R groups into targeted 25-hydroxy substrates.

Studies have demonstrated that vitamin D(3) is a natural product of a sunlight-mediated process in the skin and that its biological function is dependent on specific CYP enzymes (Omdahl et al. 2003). CYP27B1 is the regulatory rate-limiting enzyme that controls the bioactivation process and the resultant 1,25-dihydroxyvitamin D(3) (1,25D) is biologically active that directs the multitude of vitamin D-dependent actions involved with calcium homeostasis, cellular differentiation and growth, and the immune responses. The circulating and cellular level of 1,25D is governed through a coordinated process that involves the hormone's synthesis and degradation. Central to the degradation and turnover of 1,25D is the regulatory multi-catalytic CYP24 enzyme that directs the introduction of C-24R groups into targeted 25-hydroxy substrates (Omdahl et al. 2003). Thus, Vitamin D and its analogs possess a potential for novel drug development for skin diseases. The development of drugs that treat skin disease is targeted at substrates, inducers, or inhibitors of this enzyme.

The cytochrome P450 system in eukaryotes is a two-component system consisting of a single type of flavoprotein reductase and a family of related cytochrome P450 proteins. For example, there are at least 57 cytochrome P450s and 29 pseudogenes in the human genome. These genes are organized into 18 families and 43 subfamilies. Each family has a particular type of substrate specificity. For example, the CYP family 1 carries out drug metabolism, the CYP family 19 is involved in steroid biosynthesis, and the CYP family 24 deactivates vitamin D (CYP superfamily). The cytochrome P450 family also includes cytochrome P450BM-3 (a bacterial version of cytochrome P450), nitric oxide synthase (NOS), and bacterial sulfite reductase.

Like the cytochrome P450s, the cytochrome P450 reductase is a membrane-bound protein. It is comprised of three distinct domains. The amino-terminal region represents the membrane anchor. The more water soluble globular portion is composed of two major domains each binding a different riboflavin-type cofactor--the flavin adenine dinucleotide (FAD) coenzyme and the flavin mononucleotide (FMN) cofactor (Figure 1). Both cofactors are essential for the activity of the protein. This “diflavin” portion comprises the NADPH-dependent reductase activity. Cytochrome P450 reductase is one of only four mammalian proteins that are known to contain both the FAD and FMN in a single polypeptide chain (Gutierrez). Also, the rat cytochrome P450 reductase was the first enzyme in the P450 system to have its tertiary structure and domain structure established. This structure served as an aid in the determination of the structures of human and bacterial cytochrome P450 reductases. It has been recently discovered that each flavin-binding domain can be expressed as isolated proteins that retain their flavin binding properties. This is possible because the gene segments that encode each domain are contiguous within the open reading frame for the intact proteins. Each gene segment could then be amplified using the polymerase chain reaction (PCR) for cloning and expression of the individual domains.

Cytochrome P450 reductases have been under intensive investigation for many years. It is known that these enzymes can reduce cytochromes such as the P450- and c-type proteins. A homologous system has been discovered in the bacterium, *Bacillus megaterium*, dubbed the cytochrome P450BM-3 enzyme (BM3). Unlike the mammalian P450 systems, the bacterial enzyme is soluble and contains all of the redox centers, *i.e.* the flavin cofactors and the heme oxygenase domain in a single polypeptide. This enzyme functions as a monooxygenase just as the mammalian systems; however, the substrates are restricted primarily to fatty acids. For these reasons, the bacterial system has been used as an effective experimental system in which to study

the details of the reaction mechanism. The bacterial enzyme is capable of reducing other artificial electron acceptors such as cytochrome c and ferricyanide more effectively than the intact mammalian P450 reductase. The BM3 reductase is also structurally related to other mammalian diflavin reductases, including methionine synthase reductase (MSR), the isoforms of nitric oxide synthase (NOS), and the human novel reductase 1 (NR1). Despite the inferred structural similarities, the thermodynamic properties of BM3 reductase are distinct from mammalian enzymes because they show major differences in the catalytic cycles of the bacterial and mammalian enzymes. It also reduces its own heme domain at rates in excess of 200 s^{-1} , which far surpasses the reduction rates of mammalian P450s. The ability of BM3 reductase to deliver electrons rapidly to its P450 domain is due, in part, to the fast reduction of FAD by NADPH, which is considerably slower in mammalian CPR.

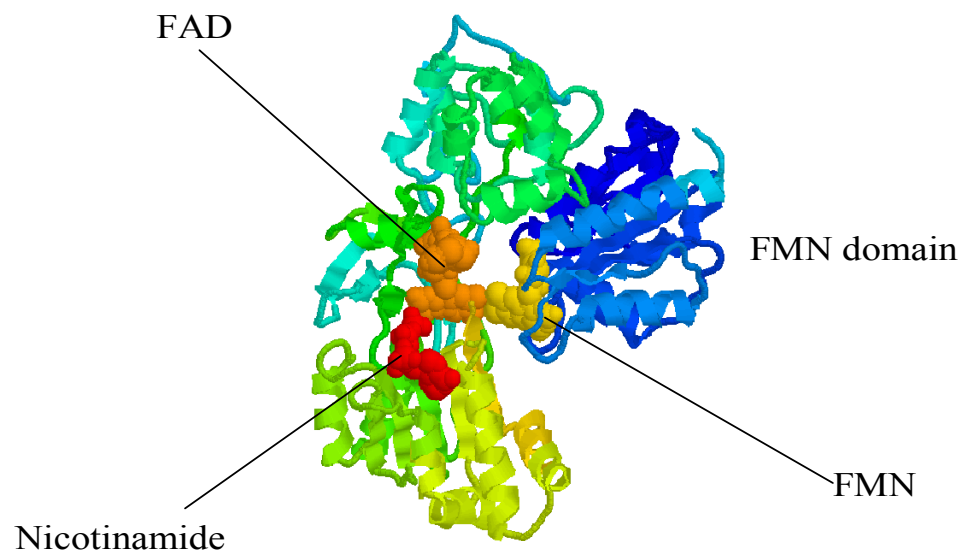


Figure 1:. Structure of cytochrome P450 reductase

Structural Properties of Cytochrome P450 Reductase: CPR has been studied with various methods such as UV-visible, EPR, fluorescence, and resonance Raman spectroscopy. The structural information was delayed, in part, due to the fact that CPR is a membrane protein. Membrane proteins are difficult to purify and to crystallize. Also, more recently the X-ray crystal structure has been determined for the rat enzyme. The structure is made up of two separate flavin-binding domains. However, it is interesting that the amino-terminal portion of the hydrophilic domain is highly exposed in the intact enzyme and is prone to proteolytic cleavage. It was discovered that this soluble domain can be released from the membrane anchor by proteolysis. When amino-terminal sequence analysis was also done on this soluble domain, it revealed that the amino-terminus residue of this domain was Ile-57. It was subsequently found that this solubilized domain could be crystallized, leading to the determination of its tertiary structure.

In the intact CPR, the FAD and FMN-binding domains are tethered to each other forming a flexible hinge domain. This hinge domain can help move towards a closed conformation where the electrons are being transferred from the FAD domain to the FMN domain. An open conformation would be present after the FMN domain has moved 10 Å to come in contact with and reduce P450. This structure feature may be of functional significance.

Although they all share an overall structural similarity, there are some structural differences between the rat FMN-binding domains, the BM3-FMN-binding domain, and the homologous bacterial flavodoxins, especially in and around the cofactor binding site. Also, the surface potentials reveal that the charge distribution in BM3-FMN dramatically differs from that of flavodoxins, and mammalian FMN-binding domain. The flavin binding site of BM3 FMN is surrounded by mainly neutral and hydrophobic amino acid residue. On the other hand, in flavodoxins and in mammalian CPRs, the conserved negatively charged amino acid residues are

clustered near the flavin. These differences may account for some of the results obtained in this study as will be discussed more below.

Electron Transfer Properties of cytochrome P450 reductase: Cytochrome P450 reductase provides an excellent system in which to study interflavin electron transfer as well has the mechanism by which flavin cofactors mediate two- and one-electron transfers, perhaps in a sequential manner. The FAD accepts two electrons from NADPH, an obligatory two-electron reductant. In contrast, the FMN transfers one electron to reduce the heme-iron in the cytochrome, an obligatory one-electron oxidant (Figure 2). The mechanisms of electron transfer are not understood precisely so another question to ask is how does cytochrome P450 reductase work? Previously, the gene segments encoding for each of the two flavin-binding domains of cytochrome P450 have been cloned and expressed (Figure 3). Furthermore, the individual domain proteins have been purified and partially characterized. The redox potentials for each half reaction have already been determined. By studying each domain separately, one is able to more easily and clearly establish the redox properties and activities of the flavins. The goal is to establish in great detail the characteristics of the individual domains and then reconstruct the mechanism of the intact reductase.

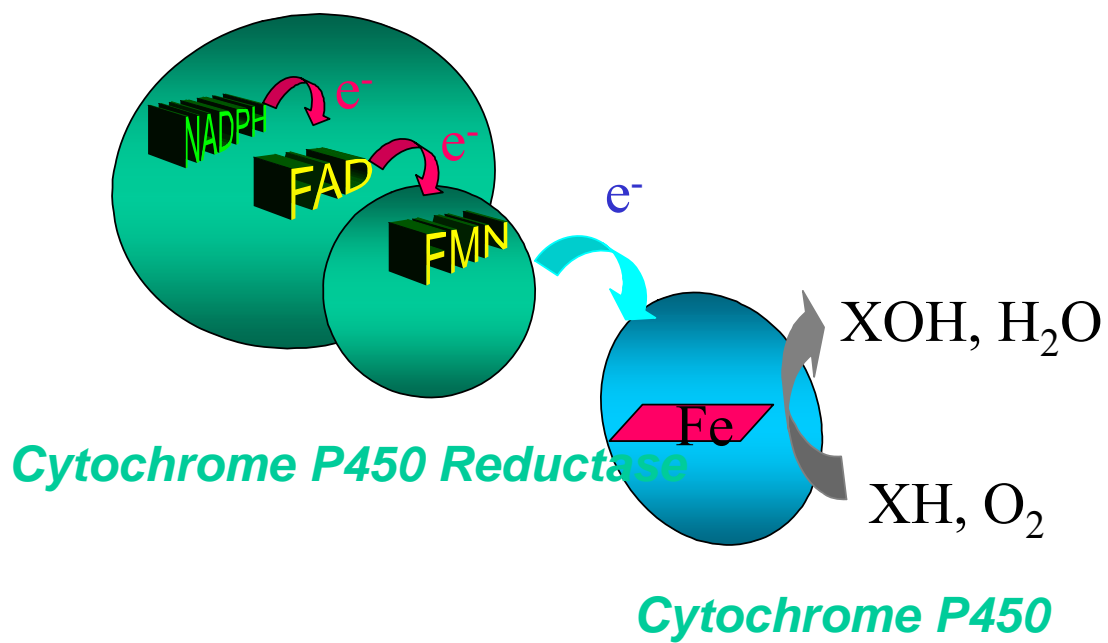
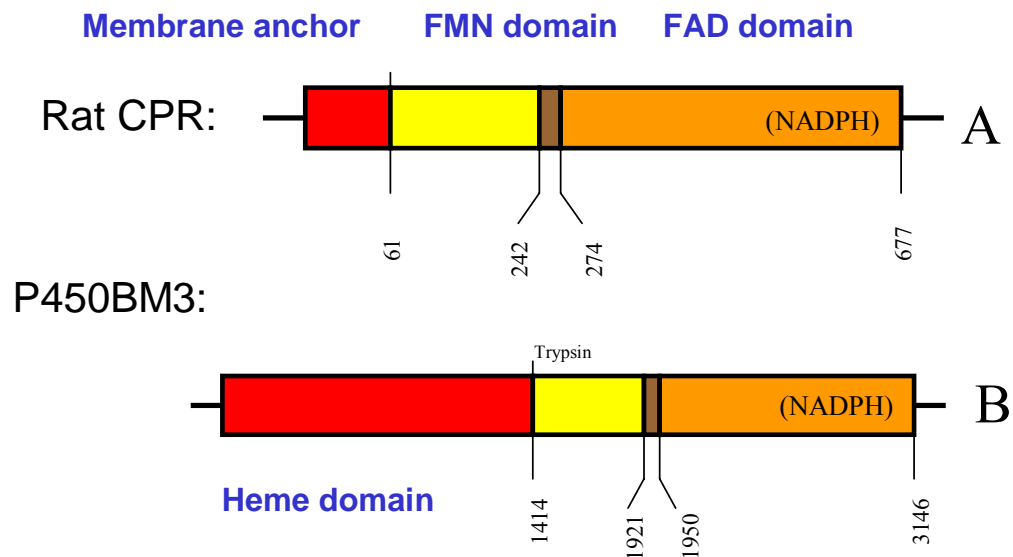


Figure 2. A proposed pathway of electron transfer for cytochrome P450 reductase



Expression of individual domains:

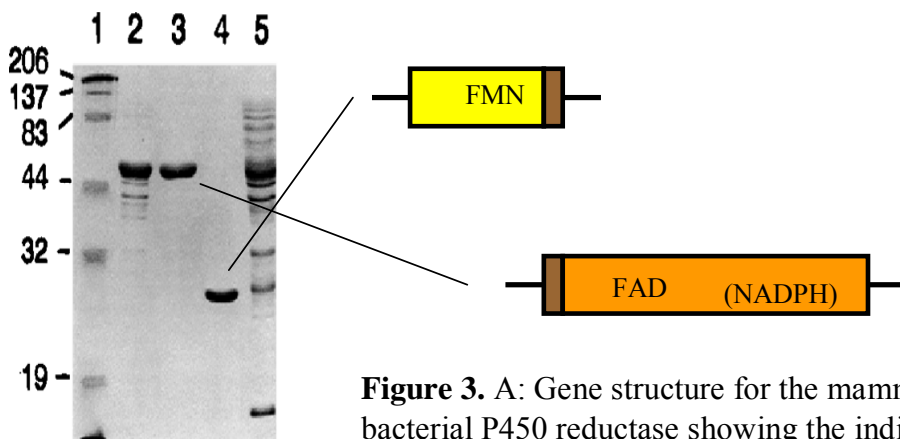


Figure 3. A: Gene structure for the mammalian and bacterial P450 reductase showing the individual regions encoding the flavin domains

B: Illustrates the goal of cloning the individual domain gene segments into a bacterial expression vector (pT7-7) for the heterologous expression of the individual domain proteins in *E. coli*.

The objective of this research project is to learn more about how inter-flavin/inter-domain electron transfer functions through the use of a reconstituted system function? First, one has to check if the separated FAD-binding domain is functional. Previously, this has been done for the bacterial domains. This first step is very important because if the separated FAD-binding domain is not functional, then it can't be reconstituted at all. The next thing is to ask is does it behave like the intact reductase? The FMN-binding domain is an important part of the electron transfer mechanism and another objective is to see if the FAD-binding require an FMN-binding domain to reduce cytochrome c. Furthermore, in the intact reductase, the two domains are bound by a “tether” or flexible linker region of approximately ten amino acids. It has been proposed that electron transfer can only occur between certain domains depending on whether the conformation of the reductase is open or closed, that is, if the FMN-binding domain can shuttle between a “closed” configuration when is adjacent to the FAD-binding domain or in the “open” configuration where the FMN cofactor is more exposed for interaction with the heme domain. If the reconstituted domains are floating freely in solution, they could bind transiently in this type of mechanism. Or, there could be a stronger interaction between the domains that might lead to a more stable electron-transfer complex. Also, the protein domains usually have specific receptor sites that each one can bind to.

In general enzymes with multi-domains could only interact with its own domains. With an intact cytochrome P450 system, it would be unknown if the FAD-binding domain can interact with other FMN-binding domains since the enzyme is made of one polypeptide. In this project the isolated FAD-binding domain could be reconstituted with different FMN-binding domains to see what effects this has on the reduction rates (Figure 4). From here, one can find out if the mammalian FAD-binding domain can be reconstituted with other FMN-binding domains. If not,

then it shows that the FAD-binding domain is very selective. This also can determine that the structure of the binding site of different FMN-binding domains may be different from the original FMN-binding domain. A test of specificity may help find out more about the electron transfer mechanism of the reconstituted reductase.

In future studies, a reconstituted system may allow for modifications in the structures of the domains by site-directed mutagenesis, followed by studies of the effect of cytochrome c reductase activity through simple reconstitution. The mutated domains may be characterized, compared, and contrasted with the properties of the intact and reconstituted reductase. This information should give a lot of information on the electron transfer mechanisms and activity of this important enzyme.

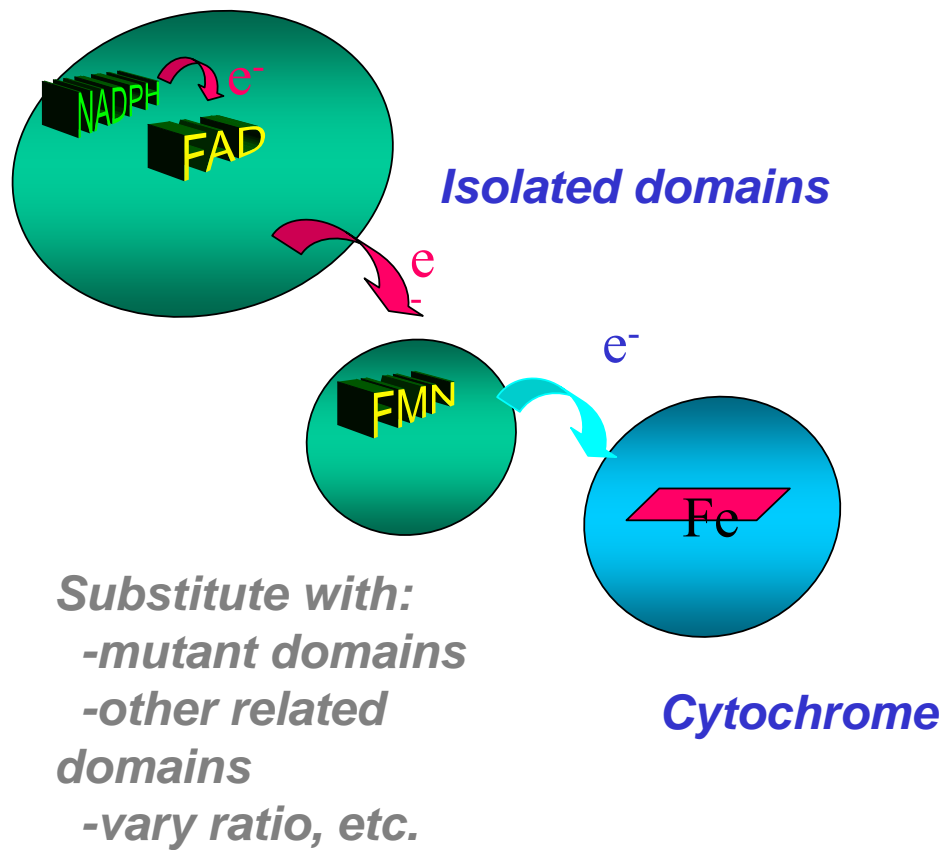


Figure 4: The reconstitution of reductase activity is done in vitro. Either domains could be substituted with different type of domains, mutants etc.

Materials:

Chemicals: Sodium dithionite, NADPH, cytochrome c, AMP, and dithiothreitol (DTT) were from Sigma-Aldrich Chemical Company. All other chemicals were also of analytical reagent grade.

Bacterial Plasmids: The BL21 Rosetta strain of competent *E. coli* cells was from Novagen and was kindly provided by Dr. Venkat Gopalan.

Rat cytochrome P450 reductase flavin-binding domains: The FMN-binding domain was purified by Ms. Yee Ling Wu. The coding region for the FAD-binding domain was cloned and inserted into the pT7-7 expression plasmid by Ms. Yee Ling Wu.

The cytochrome P450BM-3 flavin-binding domains: The FMN- and the FAD-binding domains from the soluble cytochrome P450BM-3 protein from *Bacillus megaterium* were cloned and purified by Ms. Kimara Stefanski.

Experimental Procedures:

Purification of FAD-binding domain of rat cytochrome P450 reductase: The LB growth medium was prepared by dissolving 10 gm of NaCl, 5 gm yeast extract, and 10 gm tryptone in 1 L of distilled H₂O in a 2 L Erlenmeyer flask. All flasks were autoclaved at 121°C for 20 min and cooled to room temperature. Just prior to inoculation, 4 mL of 25 mg/mL ampicillin and 1 mL of 35 mg/mL chloramphenicol were added.

Small cultures were prepared by adding 10 µL of 35 mg/mL of chloramphenicol, and 40 µL of 25 mg/mL of ampicillin to 10 mL of sterile LB. These were inoculated with a small amount of frozen *E. coli* cells (Rosetta cell line) that had previously been transformed with the pT7-7 expression plasmid containing the coding region for the FAD-binding domain from rat cytochrome P450 reductase and incubated overnight at 37°C. Five mL of these cultures were used to inoculate each of the 2 L Erlenmeyer flasks of LB. The flasks were incubated at 37°C until the OD at 550 nm reached approximately 0.8. At this time, 100 µL of 0.4 M isopropyl thiogalactoside (IPTG) was added to each flask and the cultures were then incubated at 20°C overnight. This lower incubation temperature was used in order to improve the expression of soluble holoprotein by minimizing inclusion body formation.

Cells were harvested by transferring the cell suspensions into a 1 L centrifuge bottles and centrifuging for 25 minutes. Each cell pellet was re-suspended into 20 mL of Buffer A, which contained 50 mM pH 7.7 Tris buffer, 0.1 mM EDTA, 10% glycerol, 0.05 mM DTT (added just before use). The cells were lysed with a French press at a pressure of 9000 psi. Cellular debris from the lysed cell preparation was separated by centrifugation at 12000 rpm for 30 min. The supernatant was filtered through 18.5 cm Whatman fluted filter paper for application of the first step of purification.

The FAD-binding domain was purified by affinity chromatography using a commercial preparation of agarose resin to which the ligand 2'5' ADP was covalently attached by the NH₂ group of the adenosine. A small column (1.5 cm x 8 cm) of this resin was prepared and equilibrated with Buffer A. The cellular extract was loaded onto the column followed by a wash with 150 mL Buffer A and then 250 mL of Buffer A containing 5 mM adenosine. The FAD-binding domain was eluted with 150 mL of Buffer A containing 10 mM 2'AMP and 100 mM NaCl. Column effluent was collected using a fraction collector. The purity of selected fractions containing the yellow-colored FAD-binding domain was confirmed by an SDS PAGE. The fractions that contained >98% pure protein were pooled together and the protein concentrated to approximately 1.5 mL using an Amicon stirred-cell ultrafiltration apparatus. The concentrated protein was dialyzed twice against 140 mL of Buffer A (containing 2 mg dithiothreitol) to remove the 2'AMP and NaCl.

Ferricyanide Reductase (FR) Activity Assay: Ferricyanide reductase activity directly measures the rate of the transfer of electrons from NADPH to the purified rat FAD-binding domain to the final artificial electron acceptor, potassium ferricyanide (Figure 6a). A typical assay contains 100 μ M NADPH and 500 μ M potassium ferricyanide in 300 mM potassium phosphate buffer, pH 7.7 at 25°C. The reduction of ferricyanide was monitored at 420 nm as a function of time in a UV-visible spectrophotometer.

Cytochrome c Reductase (CCR) Activity Assay: This assay measures the electron transfer from NADPH to the FAD-binding domain and the FMN-binding domain to cytochrome c. A 25 mM potassium phosphate buffer used in this assay instead of the 300 mM concentration used in the ferricyanide reductase assay because it was determined that the higher ionic strength inhibited the electrostatic interactions between the FAD-binding domain and the FMN-binding domain,

significantly reducing the electron transfer activity. Horse heart cytochrome c was used for the final electron acceptor instead of cytochrome P450 because of its commercial availability and its modest expense. The reduction of cytochrome c was monitored at 550 nm as a function of time.

Preparation of Apo-protein: 500 μ L aliquots of FMN-binding domain were each put into 2 Eppendorf tubes. Cold 10% (TCA) in 0.1 M potassium phosphate buffer and 0.6 mM EDTA (pH 7.0) was added to each tube to give a final concentration of 5% (w/v) TCA. The mixture was incubated at darkness at 4°C for 5 minutes and centrifuged at the highest speed in an Eppendorf microfuge for 10 minutes. The supernatant was discarded and the precipitate was carefully re-suspended in 5% TCA and 0.3mM EDTA for 5 min in the dark. The mixture was centrifuged for 10 minutes. This washing process was continued until the supernatant became clear. The white apoprotein pellet was dissolved in buffer and dialyzed against 100 mL of 25 mM potassium phosphate buffer, pH 7.7.

Stopped-flow Kinetics: The stopped-flow spectrometer was used to more directly measure the rates of electron transfer between, for example, NADPH and FAD-binding domain, FAD-binding domain and FMN-binding domain, or FMN-binding domain and cytochrome c. Absorbance changes as a function of time were monitored at 380 nm, 452 nm, and 580 nm. From these data, it was also possible to determine if the flavin semiquinone was formed as an intermediate and which form of the semiquinone was produced (i.e. the neutral or anionic form). Oxygen in solution can interfere with the analysis so great care must be taken to remove it. This was accomplished by flushing all reagent solutions with highly purified argon gas. Oxygen was removed from protein-containing solutions by first bubbling argon gas through the buffer component in a glass syringe followed by the addition of a small volume of highly concentrated solutions of either the FAD-binding domain or FMN-binding domain. The same potassium

phosphate buffer used in the CCR activity assay was used in the stopped-flow experiments.

Electron transfer from NADPH to the FAD domain: To determine the electron transfer rate from NADPH to FAD-binding domain, a solution contained 72 μM NADPH in 25 mM potassium phosphate buffer was mixed with a second solution containing 0.55 μM FADd, again in the 25 mM potassium phosphate buffer in the stopped flow spectrophotometer. Data were collected at 380-, 452-, and 580 nm for up to two minutes.

Electron transfer from the reduced FAD domain to the FMN domain: For this experiment, two solutions were prepared. The first solution contained 5.0 μM rat FAD-binding domain plus 200mM NADPH in the 25 mM potassium phosphate buffer. This forms the pre-reduced FAD domain under anaerobic conditions. The second solution contained 29 μM FMN-binding domain in 25 mM potassium phosphate buffer. The two solutions were mixed in the stopped-flow spectrophotometer and absorbance data accumulated for about two minutes.

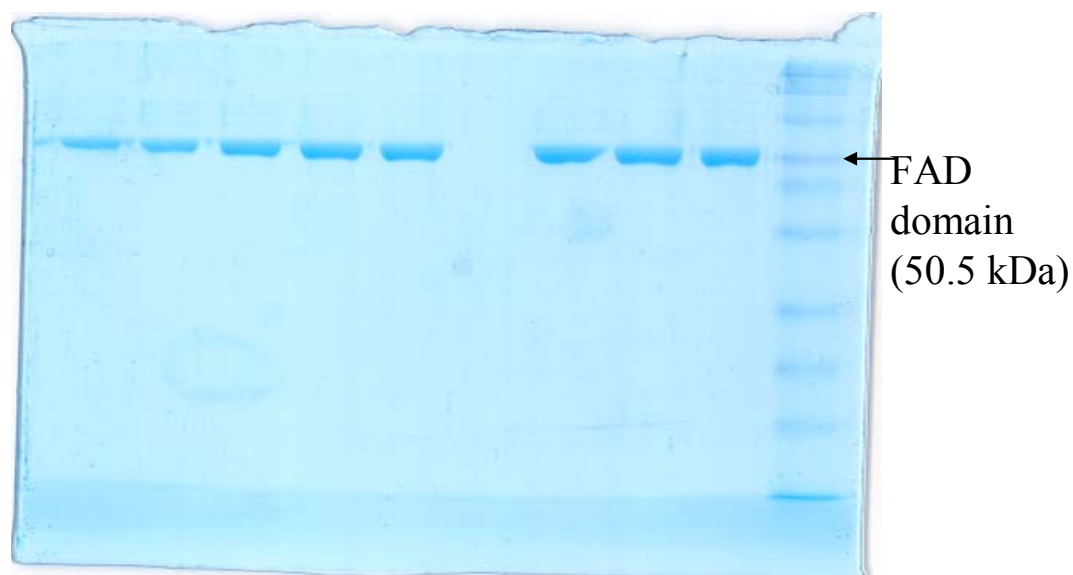
Electron transfer from reduced FMN domain to cytochrome c: To measure the electron transfer rate from reduced FMN-binding domain to cytochrome c, several factors had to be taken into account. First the FMN cofactor on the FMN-binding domain had to be reduced under anaerobic conditions. This was done by putting the FMN-binding domain into a tonometer and sparged of air by 20 cycles of argon gas and vacuum. The FMN-binding domain was reduced with sodium dithionite. The reduction of FMN was monitored with a Hewlett-Packard HP8452A photodiode spectrometer at 452 nm. It was either reduced to its semiquinone or hydroquinone state. The concentrations of the reduced FMN-binding domain was 29 μM before mixing. A set of trials were done with the semiquinone form of FMN and the other with the hydroquinone form of FMN.

Data analyses: After the experiment, the results were plotted and averaged. The

pseudo-first order rate constants were determined using either successive integration and/or the Marquadt-Levenberg fitting algorithm.

Results

Purification of FAD-binding domain of rat cytochrome P450 reductase: The protein was purified to a 98% yield. Also, it was determined that a greater yield of protein was produced when the incubation temperature was 20°C instead of 30°C. Affinity chromatography on immobilized 2' 5'-ADP showed a great efficiency in purifying the FAD-binding domain. The principle involved in affinity chromatography is that the resin contains a covalently bound ligand that will bind in a highly specific manner to the protein of interest. In this case, the 2'5'-ADP mimics a portion of the NADP(H) substrate for the FAD domain. Only this portion was necessary for good specificity which was an advantage as this resin is commercially available and relatively inexpensive. This method of purification is very efficient compared to an ion exchange column, which is not as selective. Other methods may require further steps in purification, such as running samples through more columns, which may lead to a loss of some sample. During the purification process, a dark blue band was observed to accumulate on the top of the column, indicating the reduced semiquinone state of the FAD cofactor in the domain was binding tightly to the resin. When the wash buffer was added, the band turned yellow indicating the FAD domain was converted to its oxidized state. After extensive washes, the FAD domain was eluted from the resin using 2'/3'-adenosine monophosphate. From the SDS gel, the FAD-binding domain of rat cytochrome P450 reductase was about 98% pure at this point (Figure 5).



- From right to left
1. Benchmark Marker
 2. Fraction 6
 3. Fraction 10
 4. Fraction 14
 5. Fraction 18
 6. Fraction 22
 7. Fraction 27
 8. Fraction 33
 9. Fraction 35

Figure 5: Coomassie Blue-stained SDS Polyacrylamide Gel Electrophoresis (PAGE) gel. Shown are the fractions from one step affinity chromatography.

Ferricyanide Reductase (FR) Activity Assay: The FR activity assay was successful and gave some interesting results. The activities of cytochrome P450 reductase are usually measured by two types of assays--the ferricyanide reductase activity assay and the cytochrome c reductase activity assay (see next section). The FR assay is used to measure the function of the FAD-binding domain only. This domain can promote the direct electron transfer from NADPH to the artificial electron acceptor ferricyanide (Figure 6a). In the intact protein, this process can occur without the involvement of the other parts of the protein. Thus, if the FAD-binding domain is functional, it should be able to transfer electrons from NADPH to ferricyanide without the FMN-binding domain. If the FAD-binding domain was not functional, then it couldn't transfer electrons to ferricyanide and no FR activity would be observed.

FR activity was, in fact, observed for the isolated rat FAD-binding domain. Spectral changes associated with the reduction of the ferricyanide were observed only after the addition of a small amount of the purified domain. A linear increase in the FR activity was noted as a function of added rat FAD-binding domain (Figure 6b). Another series of assays were performed at a fixed amount of the FAD-domain in the assay solution but with increasing amounts of NADPH. The initial velocity obtained from the FR assay as a function of the NADPH concentration followed Michaelis-Menten kinetics in that the curve was hyperbolic (Figure 6c). The data were fit to the equation is $y = (1.06 \times 10^{-3} \text{ } \mu\text{mol/s}[\text{NADPH}]) / (91.3 \text{ } \mu\text{mol} + [\text{NADPH}])$. A value for the K_m was determined to be 91.3 μmol . The V_{max} was found to be $1.06 \times 10^{-3} \text{ } \mu\text{mol ferricyanide/sec}$. The literature values for the intact protein show that the K_m was found to be 2.7 μM . The dependence of the FR activity at fixed concentrations of NADPH and the FAD-binding domain on the concentration of potassium ferricyanide was then determined. The assay results show that the rate of electron transfer is independent of the concentration of potassium ferricyanide shown by the

relatively flat line on the plot (Figure 6d). This was a surprising finding. Ferricyanide is only a mediator that accepts electrons from the FAD cofactor. It is likely that ferricyanide does not form an electron transfer complex, but may proceed by a second order process. It may be that electron transfer under these conditions is being limited by the rate of reduction of the FAD by NADPH, giving rise to the apparent lack of concentration dependence for the ferricyanide.

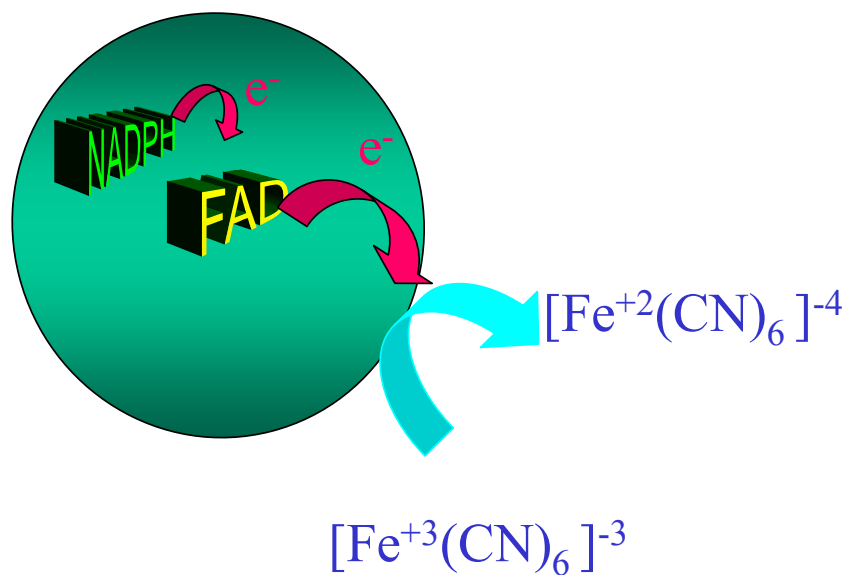


Figure 6a. The pathway of electron transfer in the ferricyanide reductase assay.

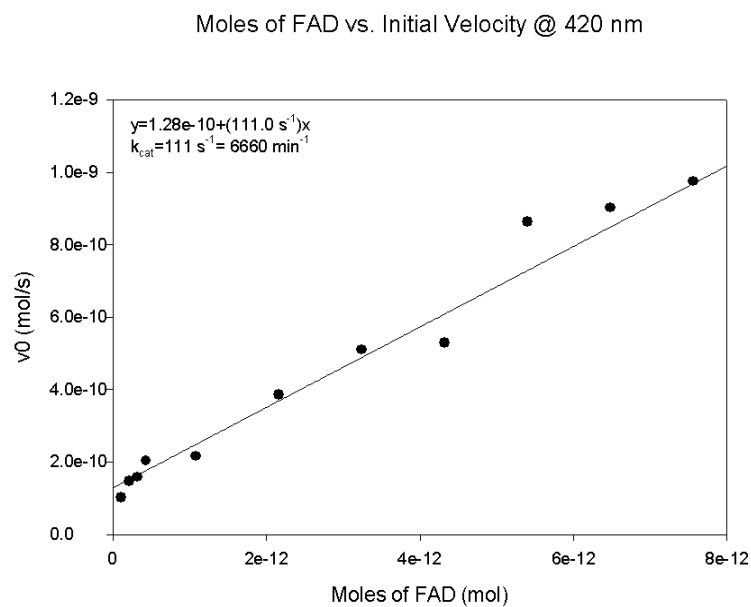


Figure 6b. The reconstituted cytochrome P450 reductase showed increasing activity as the concentration of FAD-binding domain increased.

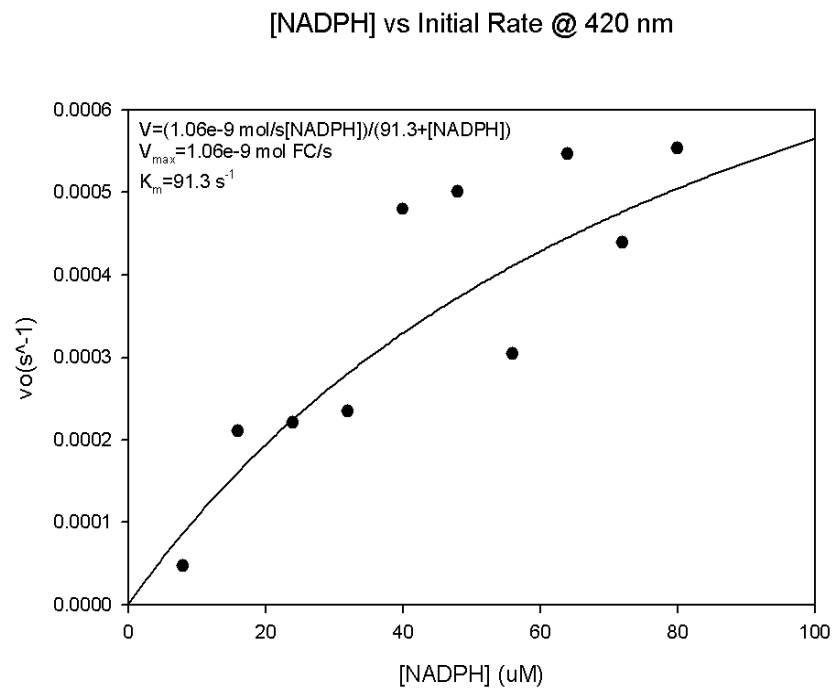


Figure 6c. The reconstituted cytochrome P450 reductase showing Michaelis-Menten kinetics as the concentration of NADPH increases.

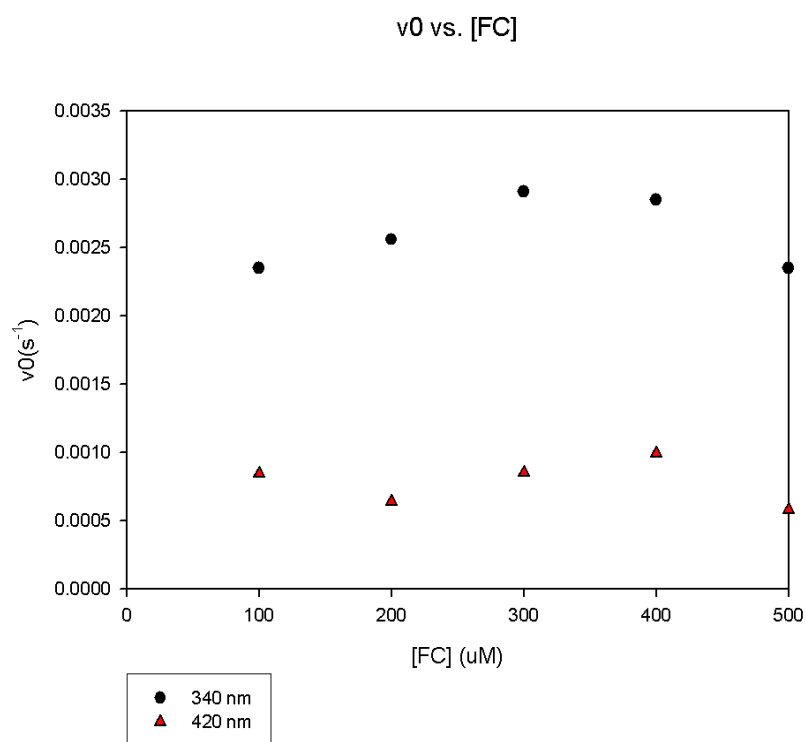


Figure 6d. The activity of the reconstituted cytochrome P450 reductase is independent of the concentration of ferricyanide (FC), an artificial electron acceptor.

Cytochrome c Reductase (CCR) Activity Assay: A second type of assay the cytochrome c reductase (CCR) assay was performed on the rat FAD-binding domain. This assay measures the electron transfer from NADPH to the FAD-binding domain and the FMN-binding domain to cytochrome c. Unlike the FR activity, the reduction of cytochrome c requires the presence of the FMN-binding domain in the intact protein and can not be catalyzed by the FAD-binding domain alone as seen in Figure 7. The next experiment was to determine if CCR activity can be reconstituted by mixing together in solution the two isolated flavin domains from the rat cytochrome P450 reductase, *i.e.* the FAD-binding domain and the purified FMN-binding domain. In this study, the ratio of the FMN-binding domain to the FAD-binding domain was varied, from 0.1 to 3.5. The rate was very low at low ratios of the FMN-binding domain, but there appeared to be an increasing linear trend as more FMN-binding domain was added. The plots for this assay increased linearly as the ratios of FMN-binding domain to FAD-binding domain increased to activity levels that were at the limits of determination. These results may indicate how the electron transfer is made between these two protein domains. Two hypotheses were made prior to the conduct of this experiment. If electron transfer involves the formation of a tight electron-transferring complex between the two flavin domains in solution, we expected that the CCR activity would start to level off after a 1:1 complex had been formed (Figures 8 and 9a). Excess FMN-binding domain would not catalyze the reduction of cytochrome c alone (as was determined in a separate experiment). However, the data are more consistent with a second hypothesis in which a transient complex is formed between the reduced FAD-binding domain and the FMN-binding domain that results in the rapid transfer of electrons to the FMN. This in turn results in a separate electron transfer reaction between the FMN-binding domain and the cytochrome c. If electron transfer between the FAD- and FMN-binding domains is very rapid,

more rapid than the second transfer to cytochrome c, then a catalytic cycling process may be involved (Figure 9b). These results demonstrate two important properties. 1) CCR activity can be reconstituted in vitro through the mixing of the two separate flavin domains. 2) CCR activity does not saturate suggesting that a tight complex between the two flavin domains is not formed, but instead, a transient electron transferring complex is formed. As will be discussed further below, this second conclusion may have important mechanistic implications for our understanding of the electron transferring process within the intact reductase protein.

Comparing Initial Rates when Different FMN domains are present

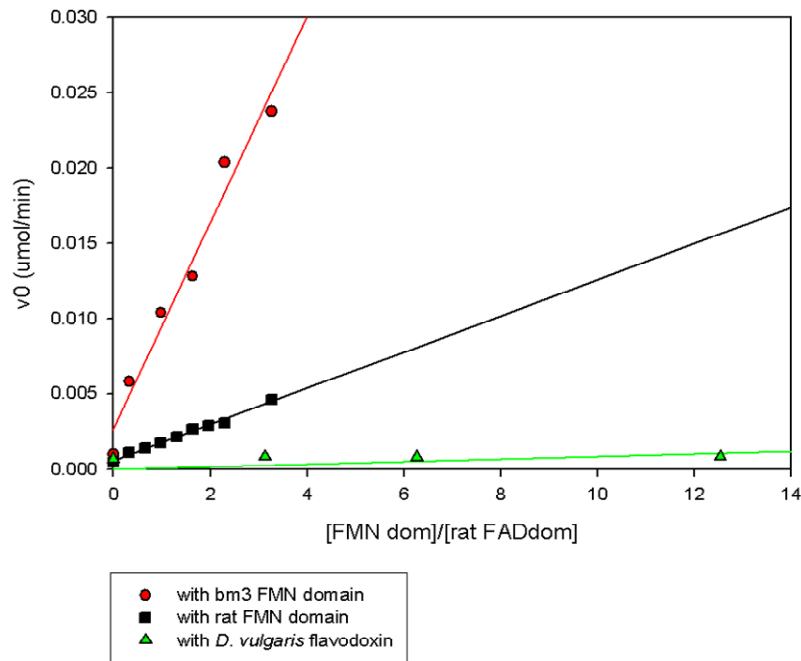


Figure 7. Cytochrome reductase activity can be reconstituted in vitro by mixing the rat FAD domain with its isolated FMN domain (■). Interestingly, significantly higher activity was obtained when the functionally equivalent bacterial FMN domain was added (●). However, very little, if any, activity resulted using the *D. vulgaris* flavodoxin (▲), a very close structural homolog of the rat FMN domain. These data demonstrate, 1) cytochrome reductase activity can be reconstituted from the two isolated flavin domains in vitro, and 2) the mammalian and bacterial CPR FMN domains are functional but not equivalent.

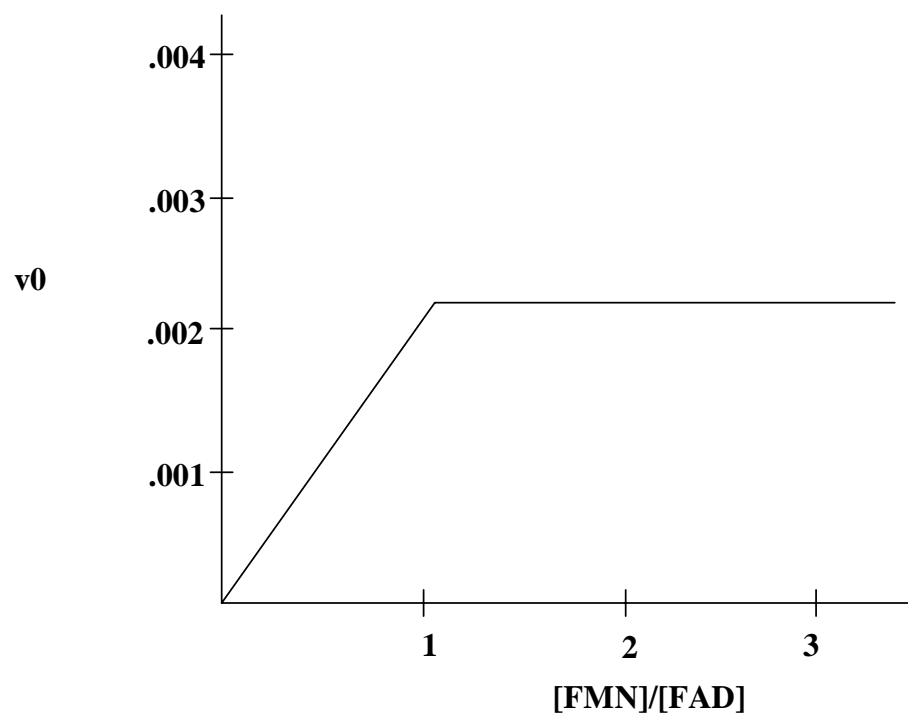


Figure 8: Theoretical graph of the rate of cytochrome c reduction which should reach a maximum at a 1:1 ratio of FMN domain to FAD domain if a tight complex forms.

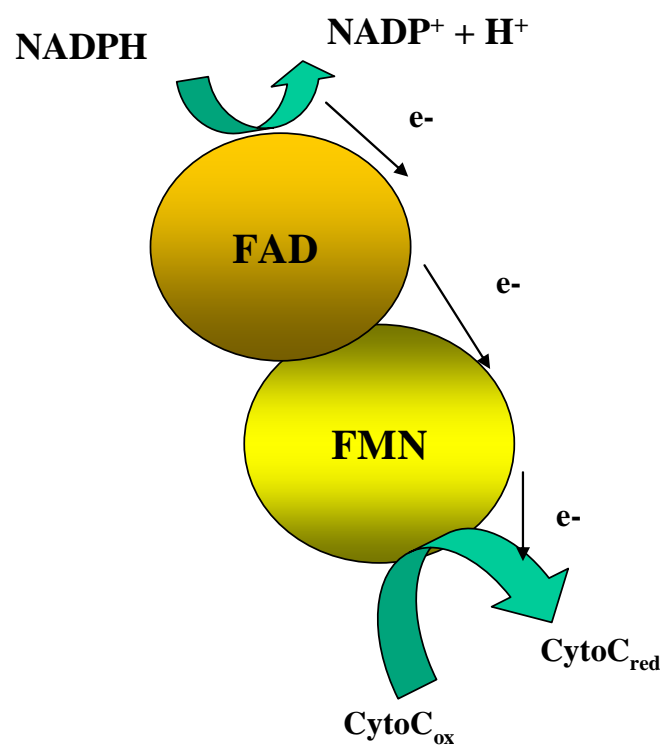


Figure 9a: Stable Noncovalent Complex Model for the Reconstituted System of Cytochrome P450 Reductase

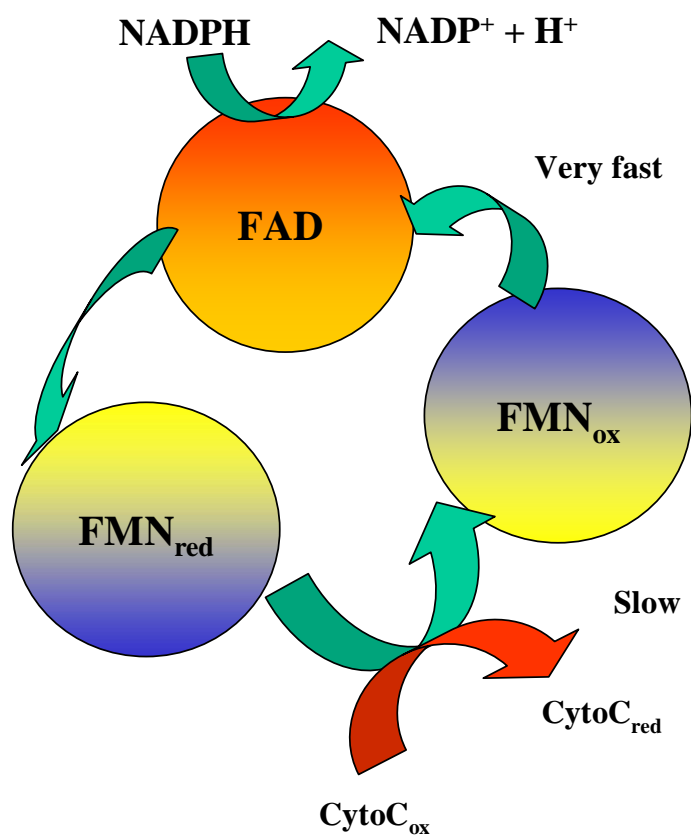


Figure 9b: Catalytic Cycling Model (transient complex) for the Reconstituted system of Cytochrome P450 Reductase

Inhibition of CCR Activity by the Apoprotein: Based on the above experimental results, a series of experiments was conducted to determine if the FMN-binding domain apoprotein would compete with FMN domain in the reconstituted CCR activity assay. The idea here is that because the apoprotein lacks the FMN cofactor, it would not be able to participate in electron transfer between the FAD-domain and cytochrome c. If a tight 1:1 complex between the FAD- and the FMN-binding domains is formed, it should be possible to observe inhibition of CCR activity by the apoprotein if it forms an inactive complex with the FAD-binding domain. When the activity was plotted as a function of the ratio of apoprotein to a constant FMN-binding domain concentration, there seemed to be no inhibition of the CCR activity. If the apoprotein could effectively compete for the same binding site on the FAD domain as for the FMN-binding domain, we expect to see a significant decrease in activity because the apoprotein, which lacks the essential FMN cofactor, could not support cytochrome c reduction. However, the plot showed a linear relationship with a very small slope (Figure 10). These data suggest that the FMN-binding domain does not form a stable complex with the FAD domain and the data may be more consistent with a catalytic cycling mechanism. Of course, there is an assumption that after the FMN cofactor was removed the apoprotein would retain its shape and be able to bind to the FAD-binding domain. This was not shown directly, however.

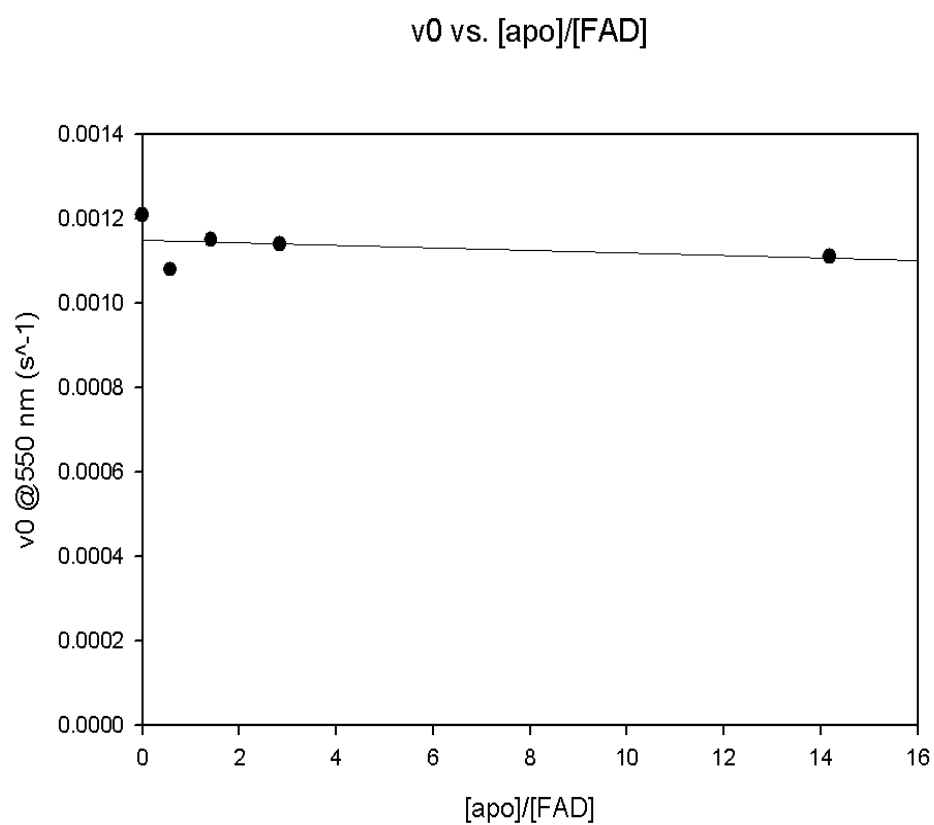


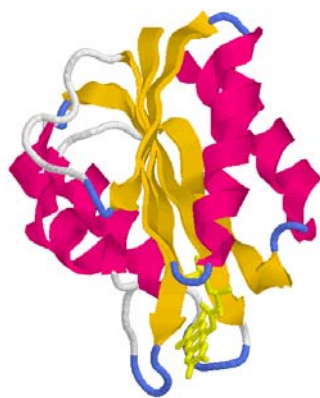
Figure 10: An apoprotein was added to see if it will compete with FMN domain to form a 1:1 complex with FAD domain. The data suggest that there isn't a significant change in the rate even if the concentration was very high

Specificity for the FMN-binding domain: Because it seems apparent that the FAD-binding domain does not make a tight complex with the FMN-binding domain in the reconstituted system, the question was asked as to how specific the transient interaction between the two flavin domains might be. Will the CCR activity depend on what type (source) of FAD or FMN binding domains that are used? In this series of experiments, the rat FAD-binding domain was tested with various FMN-binding domains to determine its specificity. The flavodoxin from the bacterium *Desulfovibrio vulgaris* is a small FMN-requiring electron transferring flavoprotein that is a close structural homolog to the FMN-binding domain from the rat cytochrome P450 reductase (Figure 11). However, the redox potentials are quite different between these two FMN-binding proteins (Figure 12). When the flavodoxin was substituted for the rat FMN-binding domain in the reconstituted system, there was little increase in CCR activity (Figure 11). The isolated FMN-binding domain from the cytochrome P450BM-3 from the bacterium *Bacillus megaterium* is a functional homolog of the FMN-binding domain in the rat cytochrome P450 reductase (Figure 11). In this case, increasing CCR activity was observed when the rat FAD-binding domain was assayed with added amounts of the BM3 FMN-binding domain. In fact, the slope of the activity versus the ratio of the BM3 FMN- to rat FAD-binding domain is about four times higher than that for the rat FMN-binding domain. This suggests that the bacterial FMN-binding domain functions more efficiently with the rat FAD-binding domain than its own FMN-binding domain. This was surprising. The corollary experiment was also performed in which the bacterial BM3 FAD-binding domain was assayed in the presence of increasing amounts of the rat FMN-binding domain. In this case, the plot showed a very small slope. However, once again, the plots were linear indicating catalytic cycling in the electron transfer (Figure 7).

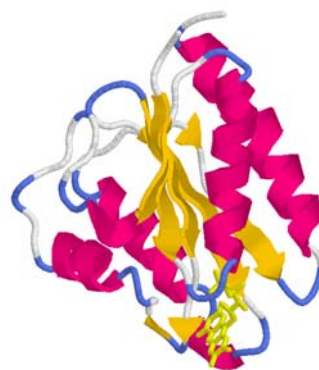
Figure 11: The FMN-binding domains/proteins that share a common α/β -flavodoxin-like structure.



FMN-domain of CPR



D. vulgaris flavodoxin
[close structural homolog]



FMN-domain of P450BM-3
[functionally equivalent homolog]

Redox Potential Control of Electron Transfer in Two P450-Systems

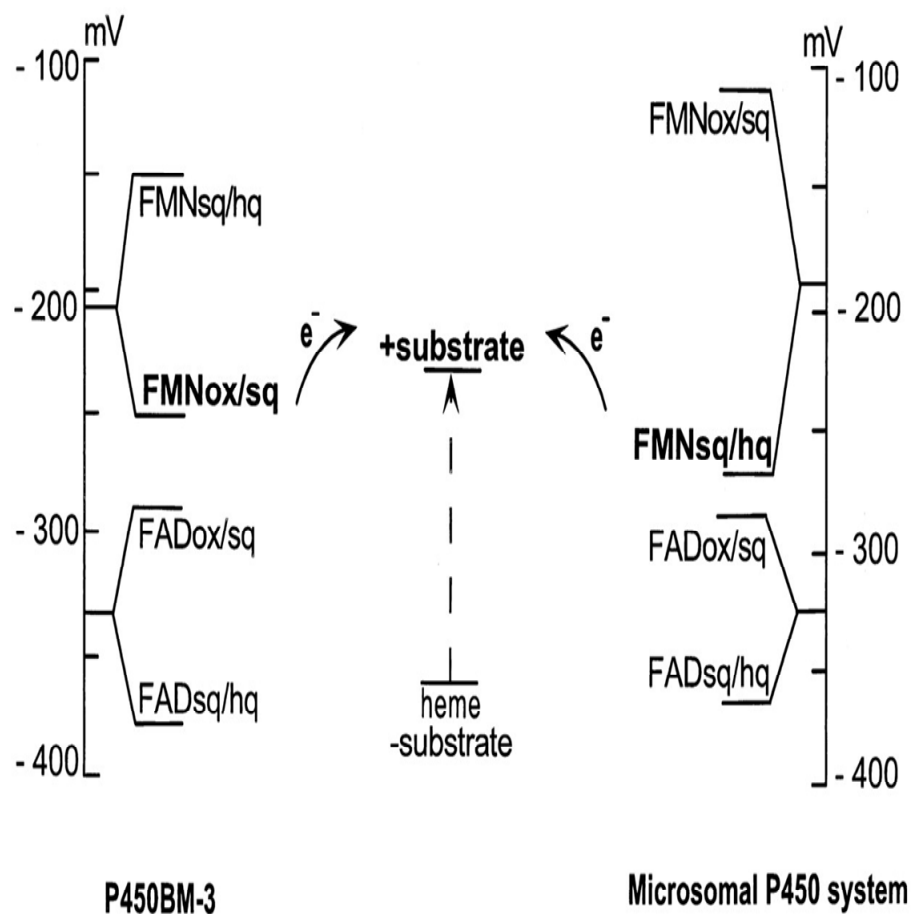


Figure 12: These are the redox potentials for the P450BM3 and mammalian P450 system.

from Sevrioukova et al. (1999) Biochemistry

Effect of domain type of cytochrome c dependency: The CCR activity as a function of cytochrome c concentration was determined while keeping either a constant 2: 1 or a 10:1 ratio of the FMN- to FAD-binding domain. The same potassium phosphate buffer used in the CCR activity assay was employed in this kind of assay. Plots indicate that the CCR activity obtained from the reconstitution of a “reductase” by mixing the BM3 FAD-binding domain with either the rat or the BM3 FMN-binding domains show hyperbolic characteristics (Figures 13 and 14). However, at higher cytochrome c concentrations inhibition of CCR activity was noted.

Comparing the data of FMN dom/FAD dom

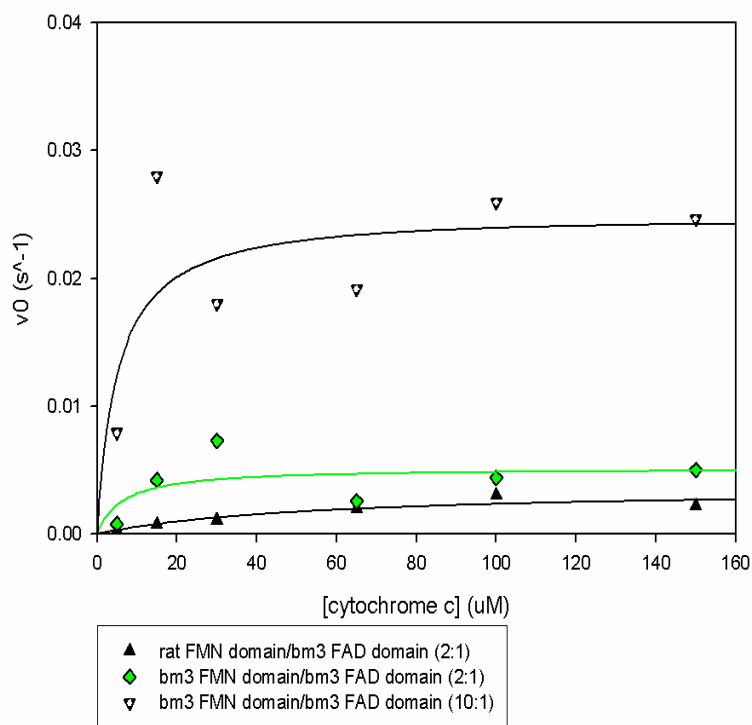


Figure 13: All of the data above were done in phosphate buffer. The BM3 FAD domain and the rat FMN domain had very little activity compared with the bm3 FMN domain and bm3 FAD domain.

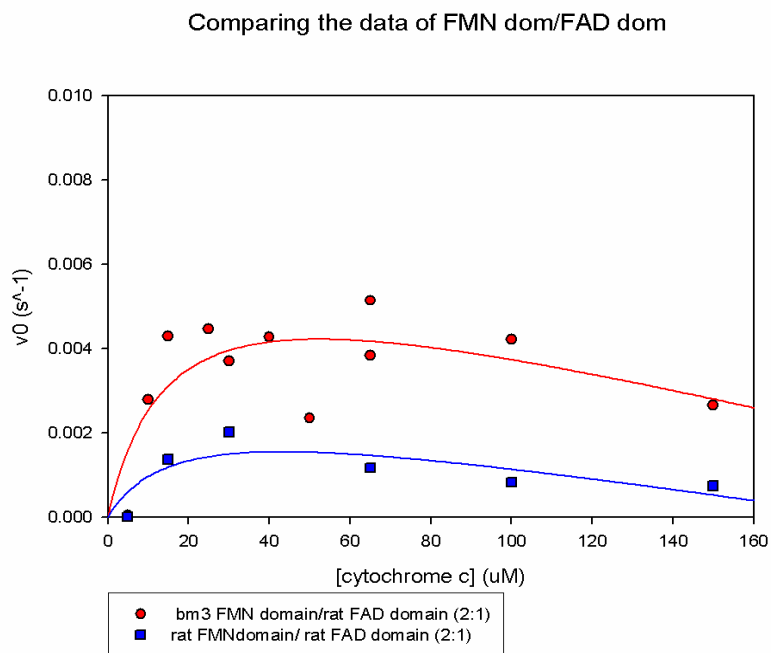


Figure 14 : The assays for the BM3FMNdomain/ratFADdomain and rat FMN domain/rat FAD domain above were done in phosphate buffer. The reconstitution of the bm3 FMN domain and the rat FAD domain show that the reduction rates are faster than the rates of the reconstituted rat FMN domain and rat FAD domain.

Effect of ionic strength on the of cytochrome c reductase activity. During the course of the above studies, it was noted that the CCR activity was dependent on the buffer type and concentration. It was reasoned that the interactions between the two flavin domains (and, thus, the activity) might be affected by ionic strength. Thus, a series of assays were set up in which the buffer was maintained as 10 mM Tris buffer, pH 7 and the ionic strength was varied by the addition of sodium chloride. CCR activity was determined to see how ionic strength influenced reconstitution. The rates showed an initial increase but at excess cytochrome c concentrations, the rates dropped (Figures 15a and 15b). Near excess cytochrome c concentrations, the curve dropped more sharply for the BM3 system than the rat system (Figure 16). The results indicated that for the BM3 system, the activity was optimized at 10 mM Tris buffer. The initial velocities of the BM3 system in the 100 mM Tris buffer were lower because the increased ionic strength may interfere with the contact between the binding sites of the two domains. The fact that both of these reconstituted systems showed inhibition with increasing concentrations of cytochrome c indicated that they may transfer electrons with similar mechanisms.

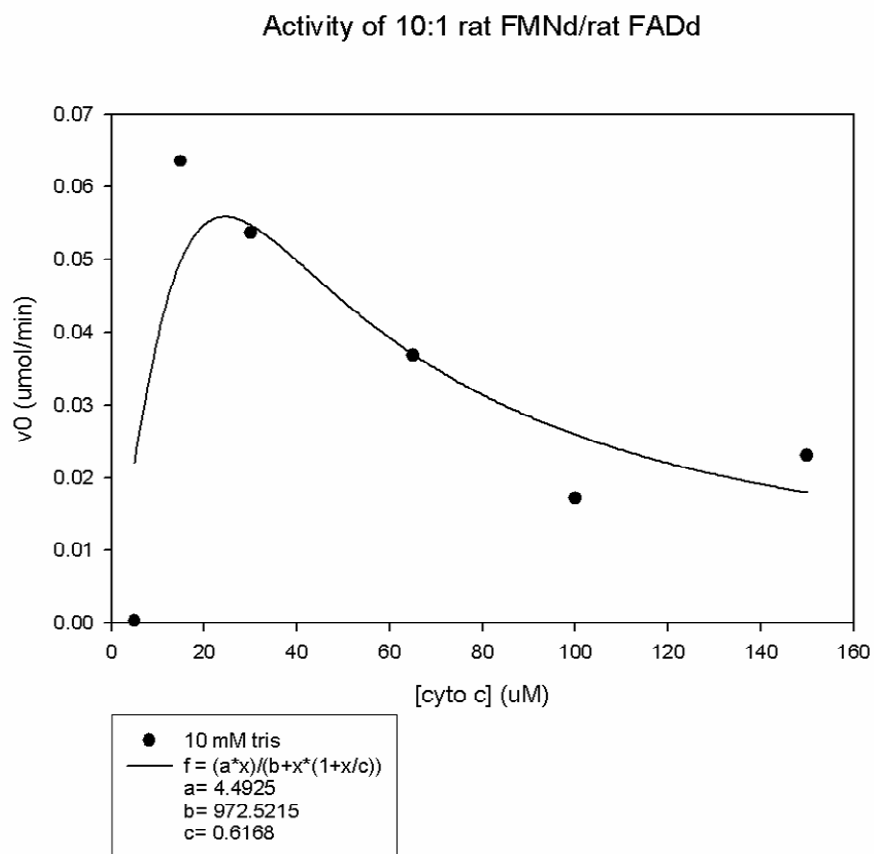


Figure 15a: The reconstituted rat cytochrome P450 reductase showed inhibition of electron transfer in 10 mM tris buffer.

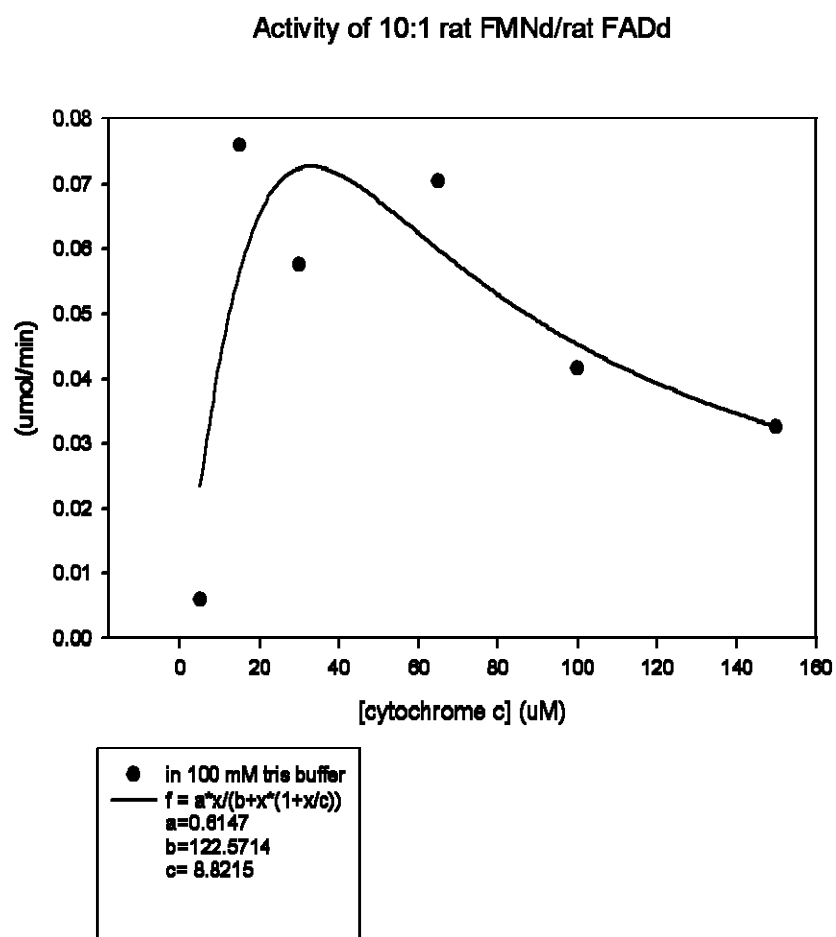


Figure 15b: The reconstituted rat cytochrome P450 reductase showed inhibition of electron transfer in 100 mM tris buffer.

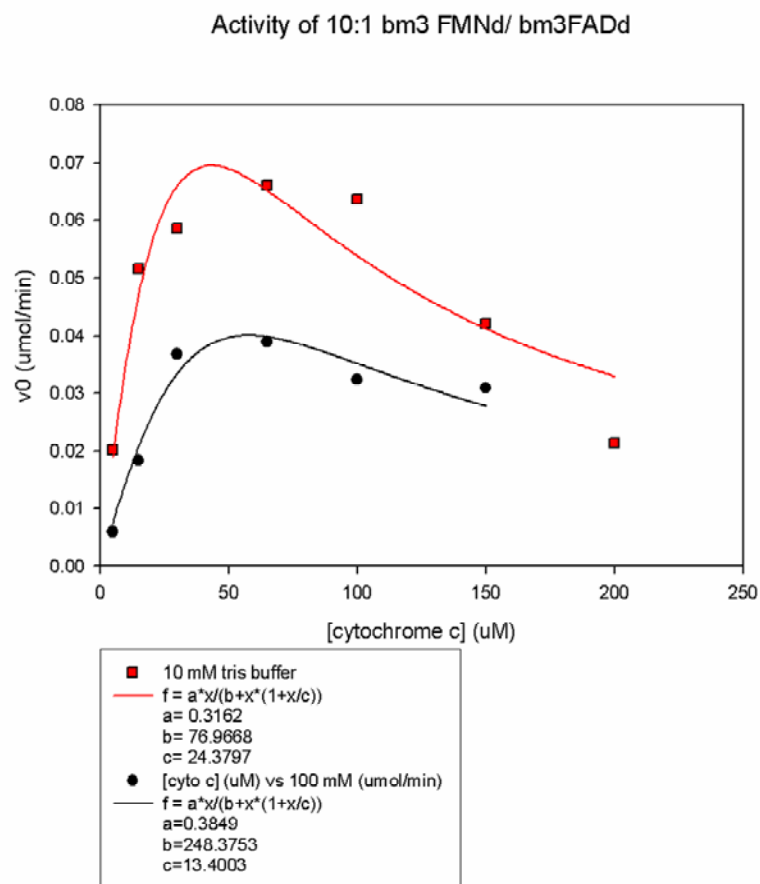


Figure 16: The reconstituted bm3 cytochrome P450 reductase showed inhibition of electron transfer in 100 mM and 10 mM tris buffer. The activity seemed to be affected by ionic strength. At lower ionic strength, the activity seemed to be much better.

Stopped-flow Spectrometry: Stopped-flow experiments were conducted to measure the electron transfer rates from NADPH to FAD-binding domain, from reduced FAD-binding domain to various FMN-binding domains, and then from reduced FMN-binding domain to cytochrome c. For most of these experiments, the rates were monitored at 452 nm, 580 nm, and 380 nm. At 452 nm, the reduction of the oxidized flavin is primarily monitored, although other redox states do contribute a small amount to the absorbance changes. The appearance of absorbance changes at 580 nm can be entirely assigned to the formation of the blue neutral flavin radical (semiquinone). Absorbance changes at 380 nm are more difficult to interpret because all redox states of the flavin absorb in this region as well as the NADPH. However, the red anionic form of the flavin radical (hydroquinone) does absorb strongly at this wavelength. Large absorbance increases in this region during the reductive reaction generally signal the formation of this intermediate.

The most straightforward experiment to perform was to measure the electron transfer rate from NADPH to the rat FAD-binding domain. Three different NADPH concentrations were employed--60 μM , 120 μM , and 240 μM . As a result the rates were 144 sec^{-1} (60 μM NADPH), 104 sec^{-1} (120 μM NADPH), 163 sec^{-1} (240 μM NADPH) using a Marquadt Levenberg algorithm. There was a single exponential phase in both of the 452 nm and 580 nm plots with the plot decreasing. At 452 nm, this indicated that there was a reduction of FAD (Figure 17). If a blue neutral flavin radical were formed, a double exponential phase would be seen at 580 nm. The absence of a two exponential phase indicated that a blue neutral flavin radical wasn't formed. Instead what could be concluded was that the FAD-domain was reduced directly to a hydroquinone.

The next step in the overall reductive pathway is to determine the rate of electron transfer from reduced FAD-binding domain to FMN-binding domain. Previous data showed that

FAD-binding domain required the FMN-binding domain to reduce cytochrome c. The reduced rat FAD-binding domain and rat FMN-binding domain were reconstituted for the stopped flow. The stopped-flow plot of the reconstitution of reduced rat FAD-binding domain and rat-FMN-binding domain was a single exponential phase at 452 nm. There was a decreasing trend in that plot showing a reduction of the rat FMN (Figure 18a). At 580 nm on the other hand, a double exponential phase was present with the plot initially increasing and then decreasing (Figure 18b). This indicated that a semiquinone was forming.

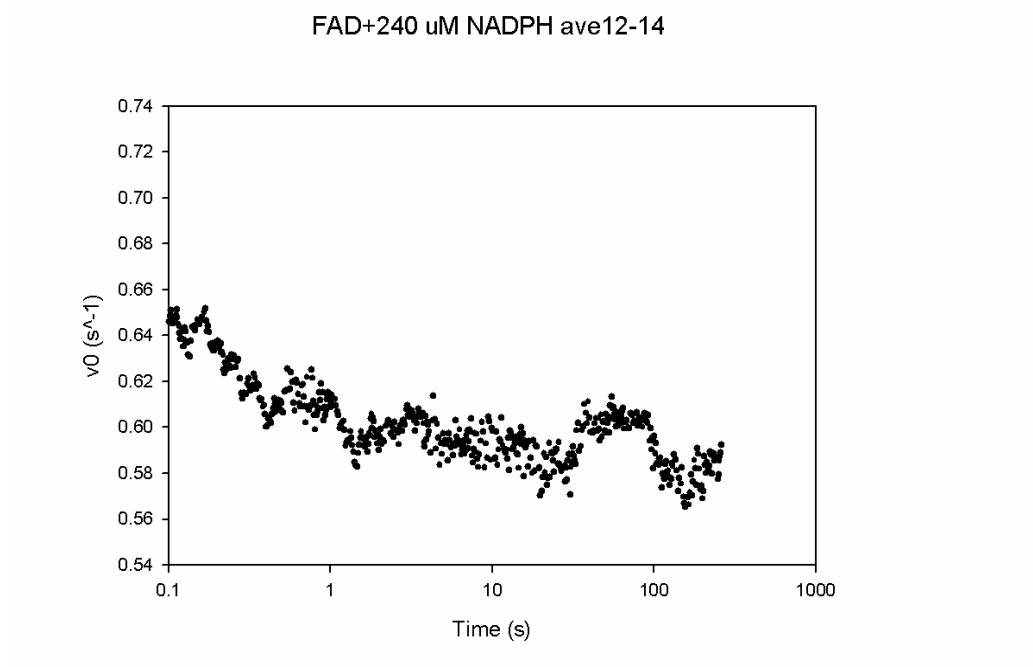


Figure 17: The NADPH is reducing the rat FAD-binding domain.
The rate was 163 s^{-1} .

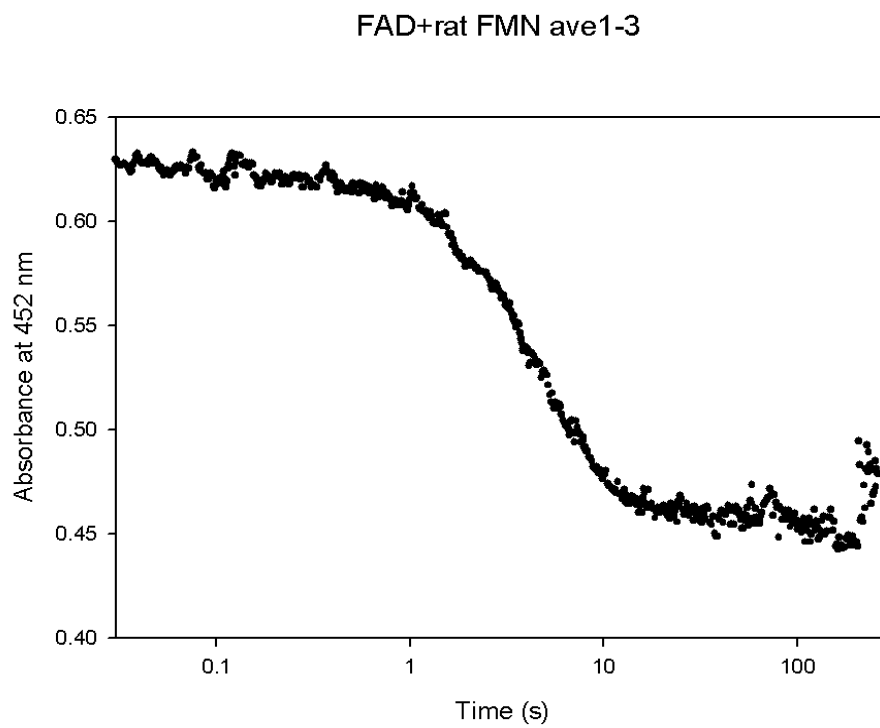


Figure18a: At 452 nm, the absorbance is decreasing suggesting a reduction of the FMN-binding domain.

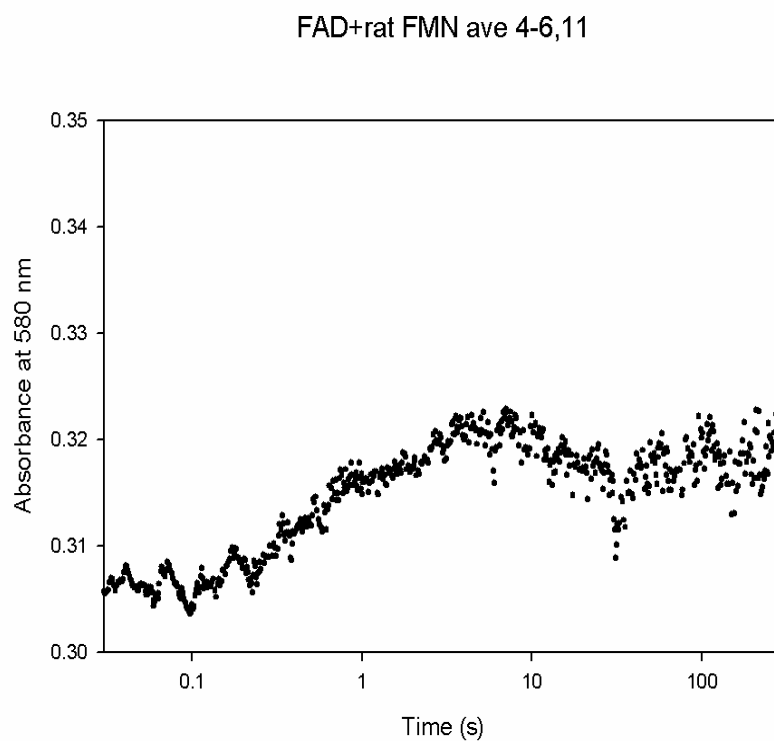


Figure 18 b: At 580, the detection of a blue neutral radical (semiquinone) was monitored. The absorbance transiently increased to a point showing a formation of a semiquinone and then it decreases, showing the appearance of the fully reduced FMN.

Stopped-flow measurements were also done with BM3 FMN-binding domain, a functional homologue. It was surprising that the combination of rat FAD-binding domain and BM3 FMN-binding domain resulted in a better electron transfer system than that of the combination of the rat FAD-binding domain and BM3 FMN-binding domain. It was unknown where the fast electron transfer was occurring. This stopped-flow experiment would help determine whether this surprisingly fast electron transfer occurred between rat FAD-binding domain and BM3 FMN-binding domain or was it between the reduced BM3 FMN-binding domain and cytochrome c. The plots of the BM3 data showed single exponential phases with a decreasing trend at both 452 nm and 580 nm (Figures 19a and 19b). The k_1 for the BM3 data was 1.19 sec^{-1} , and for the rat data, the k_1 was 0.17 sec^{-1} . The rates for the BM3 were four times higher than the rates from the reconstitution with rat FMN-binding domain. This was consistent with the steady-state assay data. The next step is to find out if the flavodoxin could transfer electrons only to FMN-binding domain but not to cytochrome c after that. Flavodoxin, which is structurally similar to the rat FMN-binding domain but with a different redox potential was reconstituted with the rat FAD-binding domain. Results showed that the reconstitution of the rat FAD-binding domain and the flavodoxin showed very little activity.

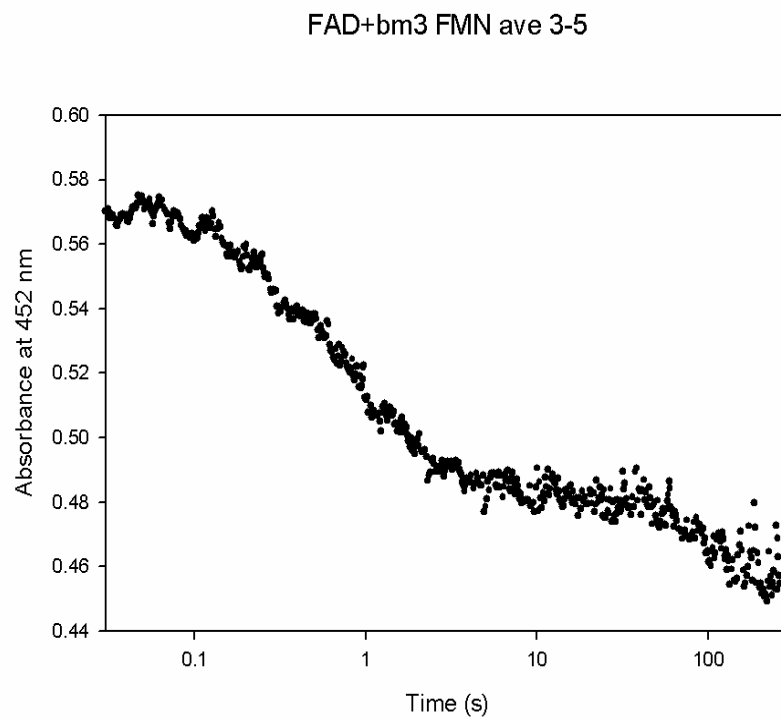


Figure 19a: At 452 nm, the absorbance is decreasing suggesting a reduction of the FMN-binding domain.

FAD +bm3FMN ave 6-8

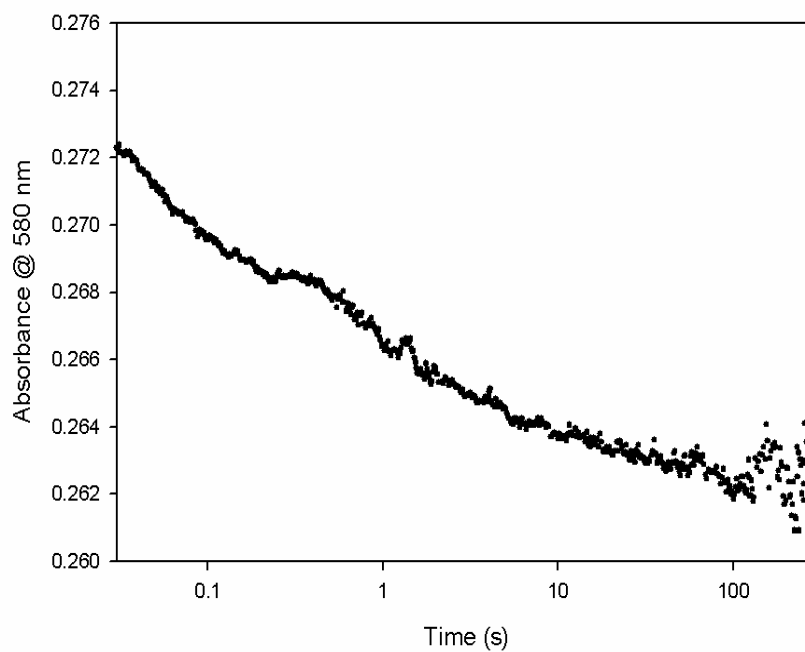


Figure 19b:At 580, the detection of a blue neutral radical (semiquinone) is monitored. The decrease in absorbance showed that the FMN was fully reduced to its hydroquinone state.

The stopped flow experiment designed to measure the electron transfer rate between the reduced FMN-binding domain and cytochrome c was the most challenging technically. Due to the complexity and experimental difficulty of these measurements, the results can only be considered to be preliminary at this point. However, some interesting observations were made. Three different cytochrome c concentrations were employed--10 μ M, 65 μ M, and 37.5 μ M. The reduction of cytochrome c was monitored at 550 nm. At 550 nm, an exponential phase was formed and the plot was increasing indicating a reduction of cytochrome c (Figure 20). At 580 nm and 452 nm a single exponential phase was found. The plots showed an increasing relationship indicating that cytochrome c was being reduced. The rates for the SQ form of the FMN were 0.28 sec^{-1} , and 0.53 sec^{-1} for 10 μ M cytochrome c and 65 μ M cytochrome c respectively. The rates for the HQ form of the FMN were 0.34 sec^{-1} and 0.77 sec^{-1} for 10 μ M and 65 μ M of cytochrome c respectively.

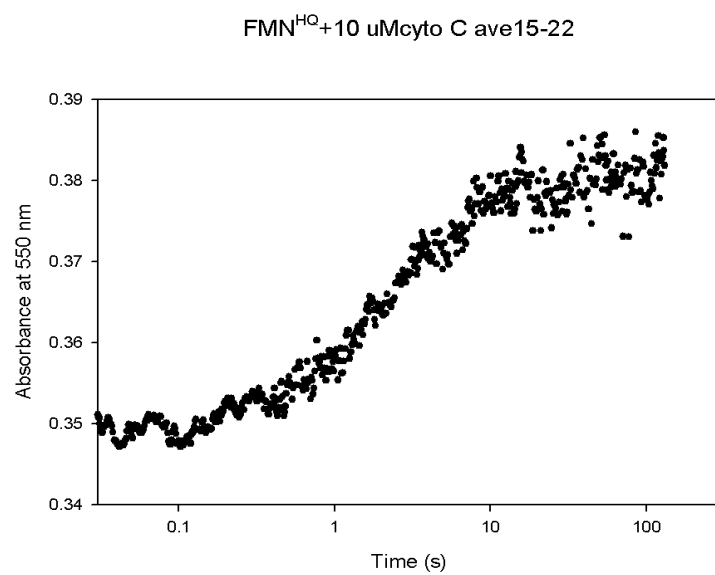


Figure 20: The reduction of cytochrome c by dithionite-reduced rat FMN domain.

Discussion:

The goals of this research project were to purify the FAD-binding domain from rat cytochrome P450 reductase, to characterize the FAD-binding domain electron transfer activity, to study the CCR reductase activity reconstituted *in vitro* from its isolated flavin domains, and to determine its specificity of the FAD-binding domain for the FMN domain for cytochrome c reductase activity. Most of these goals were attained.

The FAD-binding domain was purified to near homogeneity using an affinity chromatography as the holoprotein with the bound FAD cofactor. The electron transferring activities of this isolated domain were observed to be similar to that of the intact mammalian cytochrome P450 reductase. The NADPH-dependent ferricyanide reductase activity demonstrated that the FAD cofactor in this isolated domain remains capable of efficiently catalyzing the abstraction of a hydride equivalent from the reduced pyridine nucleotide with the ultimate transfer to ferricyanide, presumably through two rapid one-electron transfer steps involving the transient formation of the FAD semiquinone. In fact, evidence for the reductive half reaction and the rate of electron transfer from NADPH to the FAD was evaluated by stopped-flow spectrophotometry. With NADPH as the substrate, and all other variables constant, the plot of FCR activity vs. the concentration of NADPH followed Michaelis-Menten kinetics. The K_m and V_{max} were determined to be 91.3 μmol and 1.06e-3 μmol ferricyanide/sec, respectively. The concentration of ferricyanide didn't affect the electron transfer rate of CPR most likely because this reaction proceeds by a second-order process without the formation of an protein bound Michaelis complex and because other steps are rate limiting such as the reduction of the FAD by NADPH.

The studies that examined the ability of the isolated FAD-binding domain to reduce

cytochromes were perhaps more interesting. Of course, the physiological substrates for the mammalian cytochrome P450 reductase are the members of the large family of cytochrome P450 proteins. However, these proteins are difficult to purify or expensive to purchase. Thus, horse heart cytochrome c was used as a “surrogate” cytochrome in these studies. Although this is a non-physiological substrate, it has been used extensively in mechanistic studies involving cytochrome P450 reductase because it serves as a very capable electron acceptor without conducting the subsequent oxygenase chemistry which can also complicate the experimental analyses. However, it is recognized that some of what is being learned using cytochrome c may need to be confirmed using one of the natural cytochrome P450 substrates. That being said, the CCR activity assay demonstrated that the FAD-binding domain cannot directly reduce cytochrome c by itself. This was not a surprising outcome because it was known that the intact reductase cannot reduce cytochromes after the FMN cofactor had been removed. However, it was possible in those studies that the removal of the FMN caused structural changes in the reductase that affected its ability to reduce cytochromes. So, these studies with the isolated FAD-binding domain are at least consistent with the need for the FMN domain in cytochrome reduction.

The primary goal of this study was to determine if cytochrome c reductase activity can be reconstituted *in vitro* by mixing together the isolated flavin domains. If so, then these studies would demonstrate that the reconstituted system could be of tremendous value in the study of the mechanism of this complex flavoprotein reductase. The first question that we wished to address is whether the presence of the isolated FMN-binding domain could facilitate electron transfer from the FAD-binding domain to the cytochrome as has been proposed for the intact enzyme. The data clearly demonstrate that CCR activity is induced by adding increasing amounts of the isolated FMN-binding domain to the assay mixture. However, the results were somewhat unexpected.

The CCR activity was observed to increase linearly with increasing an increasing ratio of FMN-binding domain to FAD-binding domain. This relationship continued well beyond a 1:1 stoichiometry. We might have anticipated that the two domains would form a functional 1:1 noncovalent complex in order for electron transfer to occur between the NADPH and the cytochrome, much as is the case in the intact reductase. If this were happening, one would anticipate observing the saturation of the activity, that is, that the activity would level off and remain constant beyond a 1:1 ratio of the FMN-binding domain/FAD-binding domain. However, the data suggest that a catalytic cycling process may be involved. In this case, the FAD-binding domain is rapidly reduced by NADPH prior to the formation of a relatively transient complex with the FMN-binding domain during which electron(s) are transferred between the flavins. The reduced FMN-binding domain is then released by the FAD-binding domain prior to forming an electron-transfer complex with the cytochrome c. It was predicted that electron transfer from FAD-binding domain to FMN-binding domain as the slow step, and the transfer of electrons from the FMN-binding domain to the oxidized cytochrome c as the fast step.

The stopped-flow data showed that the rates for the NADPH (144 sec⁻¹ for 60 μM NADPH (final), 120 μM NADPH (104 sec⁻¹), 240 μM NADPH (163 s⁻¹)) were faster relative to the electron transfer from the rat FAD to the rat FMN (0.18 sec⁻¹) and from FMN to cytochrome c (0.34 sec⁻¹). The slowest step was the electron transfer from the FAD-binding domain to the FMN-binding domain. Previously, a catalytic cycling mechanism was proposed. In the catalytic cycling mechanism, the electron transfer from the reduced FAD-binding domain to the FMN-binding domain should be slower than the rate of reduction of the FAD by NADPH. This would account for the linearly increasing rates of cytochrome c reduction with increasing amounts of added FMN-binding domain to the steady-state assay. If the reduction of the FAD were slow

(rate limiting) then one might not expect to see this effect. Thus, the stopped-flow data mentioned above seem to be quite consistent with the catalytic cycling mechanism.

The stopped-flow data with the BM3 FMN-binding domain and flavodoxin also appear to be consistent with the steady-state data. For instance, the stopped-flow results for the reduction of the BM3 FMN-binding domain by the reduced rat FAD-binding domain displayed rates that were approximately four times faster than for the reduction of the rat FMN-binding domain by the rat FAD-binding domain. Recall that the steady-state assays indicated that cytochrome c reduction was faster in the system reconstituted with the rat FAD-binding domain and the BM3 FMN-binding domain. It was unknown if the BM3 FMN-binding domain could transfer electrons to cytochrome c faster than the rat FMN-binding to cytochrome c. This would be a useful future experiment. The fast electron rate was quite surprising because the redox properties of the rat and the BM3 FMN-binding domain are different and are thought to proceed using different mechanisms and redox couples in each case. Many more interesting experiments could be conducted on this aspect in order to learn more about these electron transfer steps.

The system reconstituted using the rat FAD-binding domain and flavodoxin resulted in little activity during the stopped-flow experiments and the steady-state assays. The stopped-flow experiment helped determine where this lack of transfer was occurring. If it were to occur between FMN and cytochrome c, then activity should be seen between the FAD and FMN. The reconstitution with rat FAD-binding domain and flavodoxin resulted in little activity during the stopped-flow experiments. It could be concluded that the electrons couldn't be transferred from the FAD-binding domain to the flavodoxin. This was not a completely surprising result because the redox properties of the flavodoxin are quite different from the cytochrome P450 reductase FMN-binding domains although they share structural similarities, especially in the case of the rat

FMN-binding domain.

This study demonstrated that the FAD-binding domain could bind certain FMN-binding domains. The assays showed that flavodoxin didn't function with the rat FAD-binding domain in reducing cytochrome c. When the rat FAD-binding domain was reconstituted with a BM3 FMN-binding domain, the cytochrome c could be reduced four times faster than with the reconstitution with a rat FMN-binding domain. Conversely, when the BM3 FAD-binding domain was reconstituted with the rat FMN-binding domain, the cytochrome c was reduced slower than with the reconstitution with a BM3 FMN-binding domain. This was quite surprising because the redox potentials for the rat FAD-binding domain are different from the BM3 FMN-binding domain. Also, the flavodoxin has similar redox properties as the SQ rat FMN-binding domain, but the rat FAD-binding domain couldn't use it. The rat FMN-binding domain can stabilize the blue neutral radical form like flavodoxin. On the other hand the BM3 FMN-binding domain doesn't stabilize the semiquinone form very well. Instead, when this binding domain is seen transiently, it appears to have to hydroquinone form. It is unknown why this was happening but maybe something in the BM3 FMN-binding domain was aiding the electron transfer. The rat FMN-binding domain didn't compete against the apoprotein even if apoprotein concentrations were very high.

The lack of competition with the apoprotein indicated that catalytic cycling was occurring, because if it were a covalent complex, inhibition would have been seen. Inhibition occurred in the reconstituted cytochrome P450 system of the BM3. Inhibition was also seen when the rat P450 system was used, but there is not as much inhibition as with the BM3 system. Seeing inhibition in both systems could indicate that the two systems are transferring electrons with a similar mechanism. It is possible that the FMN-binding domain may tightly bind the cytochrome c such

that the cytochrome c wouldn't come off the FMN. Maybe this forms a dead end complex.

These reconstitution studies of the cytochrome P450 reductase could lead to more ways to find out more about its mechanism. Maybe, the cause of the inhibition of both the bacterial and rat systems could be determined. The reconstitution study shows that the FAD-binding domain and the FMN-binding domain would temporarily come in contact with each other, and then the FMN-binding domain would come off and reduce cytochrome c. This mechanism was similar to the intact system except the two domains were tethered. The test of specificity couldn't be done with the intact reductase, but with the separated domains, one could see if the domains could accept a homolog. It turned out it could, indicating some structural similarities between the bacterial and the rat systems. From this study, both the BM3 and rat cytochrome P450 reductase reduced cytochrome c with a similar mechanism. We suggest that the catalytic cycling found in the reconstituted system is consistent mechanistically with the domain movement or "shuttling" mechanism that has been proposed for the intact cytochrome P450BM-3 system. In the modeled mechanism, the FMN-binding domain interacts transiently with the FAD-binding domain during which the FMN cofactor is reduced by the FAD. The reduced FMN domain then moves away from the FAD domain to assume a more "open" conformation which will allow for a greater exposure of the FMN and its interaction with the cytochrome in order to facilitate the reduction of the heme in the cytochrome. Further studies could be performed, perhaps in making key mutations on each domain and try to reconstitute them to see how the rates changed. The reconstituted system developed here could be of use in these types of studies.

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